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Teasaponin improves leptin sensitivity in the prefrontal cortex of obese mice

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Abstract

Scope Obesity impairs cognition, and the leptin-induced increase of brain-derived neurotrophic factor (BDNF) and neurogenesis. Tea consumption improves cognition and increases brain activation in the prefrontal cortex. Methods and results This study examined whether teasaponin, an active ingredient in tea, could improve memory and central leptin effects on neurogenesis in the prefrontal cortex of obese mice, and in vitro in cultured prefrontal cortical neurons. Teasaponin (10 mg/kg, intraperitoneal) for 21 days improved downstream leptin signaling (JAK2 and STAT3), and leptin's effect on BDNF, in the prefrontal cortex of high-fat diet (HFD) fed mice. Prefrontal cortical neurons were cultured with teasaponin and palmitic acid (the most abundant dietary saturated fatty acid) to examine their effects on neurogenesis and BDNF expression in response to leptin. Palmitic acid decreased leptin's effect on neurite outgrowth, postsynaptic density protein 95, and BDNF expression in cultured cortical neurons, which was reversed by teasaponin. Conclusion Teasaponin improved the leptin sensitivity of prefrontal cortical neurons in obese mice or when treated by palmitic acid. This in turn increased BDNF expression and neurite growth. Therefore, teasaponin supplementation may be used to prevent obesity-associated neurodegeneration and improve cognitive function.

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Teasaponin improves leptin sensitivity in the prefrontal cortex of obese mice

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ABSTRACT

Scope: Obesity impairs cognition, and the leptin-induced increase of brain-derived neurotrophic factor (BDNF) and neurogenesis. Tea consumption improves cognition and increases brain activation in the prefrontal cortex.

Methods and results: This study examined whether teasaponin, an active ingredient in tea, could improve memory and central leptin effects on neurogenesis in the prefrontal cortex of obese mice, and in vitro in cultured prefrontal cortical neurons. Teasaponin (10mg/kg, intraperitoneal) for 21 days improved downstream leptin signaling (JAK2, and STAT3), and leptin's effect on BDNF, in the prefrontal cortex of HFD fed mice. Prefrontal cortical neurons were cultured with teasaponin and palmitic acid (the most abundant dietary saturated fatty acid) to examine their effects on neurogenesis and BDNF expression in response to leptin. Palmitic acid decreased leptin's effect on neurite outgrowth, post-synaptic density protein 95 and BDNF expression in cultured cortical neurons, which was reversed by teasaponin.

Conclusion: Teasaponin improved the leptin sensitivity of prefrontal cortical neurons in obese mice or when treated by palmitic acid. This in turn increased BDNF expression and neurite growth. Therefore, teasaponin supplementation may be used to prevent obesity-associated neurodegeneration and improve cognitive function.

Keywords: teasaponin, obesity, leptin, neurogenesis, BDNF, cortex

1 Introduction

Recent epidemiological studies have demonstrated that obesity increases the incidence of cognitive decline in neurodegenerative diseases, such as vascular dementia [1, 2]. Empirical evidence has linked high-fat diet (HFD)-induced obesity with impairments in learning and memory, including a decline in recognition memory [3-5]. For example, HFD for 8 weeks impairs spatial learning in the eight-arm radial maze test, prior to metabolic alterations linked to obesity developing and without significant changes in plasma glucose and insulin levels [4]. HFD treatment of juvenile rodents impairs long-term spatial reference memory in the Morris water maze, without affecting acquisition or short-term memory [5]. Furthermore, in our previous study, recognition memory was impaired in chronic HFD fed mice as assessed by performing a novel object recognition test [3]. The prefrontal cortex plays an important role in higher cognitive function [6]. Abnormal structure and dysfunction in the prefrontal cortex is associated with cognitive decline and the pathogenesis of mood disorders [7-9]. Furthermore, positron emission tomography has shown that obese women have reduced activation in the prefrontal cortex in response to a meal than lean women [10]. In preclinical animal studies, a high-fat diet reduces synaptic plasticity in the prefrontal cortex [11], which leads to learning and memory impairments [12]. Moreover, obese patients are leptin resistant and have significantly higher levels of plasma leptin, which correlates with body fat mass [13]. Our previous study showed that in rodents a high saturated fat diet induces body weight gain followed by the development of obesity, which is accompanied by peripheral and then brain leptin resistance [14]. Despite this, therapeutic interventions targeting HFD-induced cognitive impairment and central leptin resistance are lacking.

The adipocyte-secreted hormone leptin has important effects on synaptogenesis and dendritic morphology in the brain, which regulate energy homeostasis and facilitate learning and memory [15-17]. For example, in vitro, leptin increases neurite outgrowth marker and synaptogenesis markers in mouse H19-7 HN neural cell lines, as well as stimulating hippocampal neurogenesis by increasing cell proliferation and differentiation [18]. Leptin replacement at physiological doses for two years substantially increased the rate of development in most neurocognitive domains in dividual with leptin gene mutation [19]. Brain-derived neurotrophic factor (BDNF) plays an important role in synaptic plasticity and neurogenesis [20, 21], cognitive function [22] and energy metabolism [23] through the activation of its receptor, tropomyosin-related kinase B (TrkB). BDNF is broadly expressed in the developing and adult mammalian brain, particularly in the cerebral cortex, hippocampus, hypothalamus and brainstem [21]. Plasma BDNF levels are positively correlated to memory performance in female patients with major depressive disorder [22]. Leptin administration significantly increases hippocampal BDNF in control but not HFD-induced obese mice suggesting decreased central leptin sensitivity in obese mice. [15]. Altogether, this evidence suggests that leptin activation of BDNF in the central nervous system may be involved in cognition, while brain leptin resistance could contribute to obesity related cognitive decline.

Evidence from clinical and animal studies show that tea has anti-obesity effects [24, 25] and improves cognitive function [26, 27]. Treatment with 1 % tea extract in diet for 6 weeks decreased body weight and adipose mass in genetically obese (ob/ob) mice [24]. Increased consumption of green tea was associated with a lower prevalence of cognitive impairment in a cross-sectional study in 1003 Japanese [26], and longitudinal analysis of data from 1438 Chinese subjects [27]. Phenolics and saponin are the two major active components extracted from tea.

Phenolics in tea have been widely investigated in previous studies, while teasaponin has received little attention. Chemically teasaponin belongs to the oleanane-type pentacyclic triterpene saponins, whose amphiphilic nature enables them to intercalate into the cell membrane and interact with cell membrane molecules to regulate downstream signaling cascades [28, 29]. Our previous study found that teasaponin significantly improves impaired leptin signaling in the hypothalamus of HFD fed mice, but did not elicit malaise [30]. In the current study, we expanded these findings to investigate the effect of teasaponin on recognition memory and leptin signaling in the prefrontal cortex of HFD fed mice. We further investigated the effect of teasaponin in cultured primary prefrontal cortical neurons in response to leptin and the saturated fatty acid, palmitic acid.

2 Materials and methods

2.1 Animals. C57Bl/6J male mice (10 weeks old, body weight: 19.6 ± 1.4 g) were obtained from the Animal Resources Centre (Perth, Western Australia), and housed in environmentally controlled conditions (temperature 22 °C, 12 hour light/dark cycle). Lab chow (LC) served as the low-fat control diet (5% fat, Vella Stock Feeds, Doonside, NSW, Australia) and was provided *ad libitum* except where noted. Mice were acclimatised for one week prior to experimentation. All procedures were approved by the Animal Ethics Committee, University of Wollongong, NSW, Australia, and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.2 Intraperitoneal (ip) teasaponin treatment. Mice were placed on the HFD (containing 60% fat by calories, Specialty Feeds, Western Australia) for 16 weeks. The animals were then

randomised into two groups, and administered either teasaponin (10 mg/kg) or vehicle (saline) i.p. injections daily for 21 days. Age-matched, LC diet control mice were maintained on the lab chow diet. Body weight and food intake were measured every two days. Teasaponin (96%, $C_{57}H_{90}O_{26}$, MW=1200) was purchased from the Aladdin Chemistry Co. Ltd, China.

2.3 Intraperitoneal glucose tolerance test (IPGTT). On day 18 of the teasaponin treatment, the mice were fasted overnight and given an ip injection of glucose (0.5g/kg) as reported previously [31-33]. Blood samples were taken from the tail vein and blood glucose measured using a glucometer (Abbott Diabetes Care, Alameda, CA) at 0 (fasting), 30, 60 and 120 min after glucose administration.

2.4 Central leptin sensitivity test. After intraperitoneal teasaponin treatment for 21 days, mice were anaesthetised and placed in a stereotactic device. A intracerebroventricular (icv) cannula was implanted into the right lateral brain ventricle (0.25 mm posterior and 1.0 mm lateral relative to Bregma and 2.5 mm below the surface of the skull) [34]. Five days after implantation, mice were fasted for 6 hours and administered either leptin (0.1 μg/3 μl) or saline (3 μl) injected into the lateral ventricle through the cannula. Food intake and body weight were measured 24 hours after the leptin or vehicle injection to examine central leptin sensitivity.

2.5 Blood and tissue collection. Four days after the first central leptin sensitivity test, the mice received a second icv leptin or saline injection and were sacrificed one hour later. Plasma and brain tissue were collected and stored at -80 °C for further analyses as detailed below.

2.6 Measurement of plasma leptin and insulin. Plasma leptin and insulin were measured using the mouse metabolic magnetic bead panel kit (Merck Millipore, MA, USA).

2.7 Western blot analysis. Protein expression in frozen prefrontal cortex (equivalent to the prelimbic cortex, PrL, dissected at the level of Bregma 2.8 mm to 1.98 mm) was determined using western blot as described in our previous study [30]. The following antibodies were used: BDNF, TrkB 145 and 95, pJAK2 and pAkt (Santa Cruz, CA, USA); pTrkB 95 (Sigma-Aldrich, St Louis, MO, USA); and pSTAT3, pGSK3β and pFOXO1 (Cell Signaling Technology, Beverly, MA, USA). Bands corresponding to the proteins of interest were scanned and band density analysed using the Quantity One automatic imaging analysis system (Bio-Rad Laboratories, Hercules, CA, USA). All quantitative analyses were normalised to β-actin, based on our previous studies [30]. Due to the small amount of tissue in the prefrontal cortex, we used a previously described modified multi-strip western blot [30, 35].

2.8 Oral teasaponin treatment. Mice were placed on a HFD for 8 weeks. The animals were then randomised into two groups, continuation of HFD for 6 weeks, or HFD with 0.5% teasaponin for 6 weeks. Age-matched, LC diet control mice were maintained on the lab chow diet. A novel object recognition test (detailed in 2.9) was performed 5 weeks after commencing teasaponin treatment. Three days after the novel object recognition test, an IPGTT test was performed as described in procedure in 2.3.

2.9 The novel object recognition test. This test is based on the innate tendency of rodents to differentially explore novel objects over familiar ones as previously described [36], with minor

modifications. In brief, the experimental procedure consisted of habituation, training and retention sessions. On day 1, for habituation, mice were placed into an open-field box (55×55cm × 35cm high) for 10 minutes with a 40 W light bulb in a sound proof room. On day 2, during the training session, two identical objects (A) were placed at opposing corners of the box, 5cm from the adjacent wall. Each mouse was then placed in the middle of the open-field box and left to explore the objects for 10 minutes. Ninety minutes later, in the retention session, one familiar object (A) was replaced with one novel object (B). Each mouse was placed in the middle of the open-field box, and left to explore for another 10 minutes. The exploration time for the familiar and the new objects was recorded. Memory was operationally defined by the discrimination index for the novel object (DI) as the proportion of time animals spent investigating the novel object minus the proportion spent investigating the familiar one in the testing period in the retention session [Discrimination Index = (Novel Object Exploration Time/Total Exploration Time)–(Familiar Object Exploration Time/Total Exploration Time)×100].

2.10 Prefrontal cortical neuronal cultures and treatment. Prefrontal cortical neuronal cultures were prepared from postnatal day 1 mice as described previously [37]. Cells were plated at a final density of $5 \times 10^5/cm^2$ into Poly-D-Lysine-coated 24-well culture plates for RT-PCR analysis. For neurogenesis imaging, cortical neurons were cultured on Poly-D-Lysine-coated coverslips. At 7 days *in vitro*, teasaponin and/or palmitic acid (P5585, Sigma-Aldrich) were added to the cultures. The method for dissolving palmitic acid was as described by Ross et al [38]. Leptin (100 ng/ml) was applied to the neuron cultures for 4 hours, followed by 44 hour exposure to teasaponin (20 or 40μM) or palmitic acid (10 μM).

2.11 Immunofluorescence and image analysis. For immunocytochemical staining, neurons were fixed with 4% paraformaldehyde and 4% sucrose in Dulbecco's PBS for 20 min at room temperature. The samples were incubated with PBS containing 0.2% Triton X-100 for 5 min, and blocked with 10% horse serum in PBS for 1 h at 37 °C. Then, either anti-BDNF antibody, anti-microtubule-associated protein 2 (MAP2) antibody, anti-synaptophysin (SYN) antibody, and anti-post-synaptic density protein 95 (PSD 95) antibody were applied overnight at 4 °C. MAP2 was visualised by goat anti-mouse IgG (H+L) secondary antibody conjugated to Alexa Fluor 594. BDNF, SYN and PSD95 were visualised with isotype-specific donkey anti-rabbit IgG (H+L) secondary antibody conjugated with Alexa Fluor 488. The origin and concentration of antibodies were given in the Table S1 for details. A fluorescent microscope (Axiovert 200, Carl Zeiss, Oberkochen, Germany) and digital camera were used to obtain images.

2.12 Neurite outgrowth and branching analysis. An open source toolkit "NeuriteQuant V1.23" (http://www.ccb.tu-dortmund.de/groups/CB/bastiaens/dehmelt/NeuriteQuant/) was installed as a plugin for Image J 1.40g (http://rsbweb.nih.gov/ij/download.html). The image processing algorithm for quantification of neuronal morphology by NeuriteQuant has been described previously [39].

2.13 Quantitative PCR. Quantitative real-time PCR (qPCR) was performed as previously described [30]. The primers used are listed in Table S2. Amplification was carried out with 45 cycles of 95 °C for 10 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds. The mRNA expression levels for BDNF, MAP2, SYN and PSD95 were normalised to γ-actin, which served as the internal control. Experiments were performed in triplicate.

2.14 Statistical analysis. Data were analysed using the SPSS 19 statistical package (SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA) and the *post-hoc* Tukey-Kramer honestly significant difference (HSD) test were used to analyse the novel recognition test, body weight, visceral fat, liver weight and the glucose tolerance test and leptin signaling of the LC, HFD, and teasaponin treatment groups in response to saline and leptin, as well as data from the cell culture experiments. A p value < 0.05 was regarded as statistically significant. Values are expressed as mean±SEM.

3 Results

3.1 Teasaponin prevented weight gain, fat deposition and improved glucose intolerance in HFD fed mice.

Teasaponin treatment (ip injection) significantly reduced body weight gain (Fig 1A). The final body weight of the teasaponin treatment group was significantly lower than that of HFD mice, but still higher than LC group (Fig B). As shown in Fig 1C, treatment with teasaponin significantly decreased average energy intake in HFD fed mice. Teasaponin treatment also significantly decreased epididymal fat and liver weight in animals maintained on a HFD diet (Fig 1D and E). In the IPGTT, blood glucose levels were lower at the 0, 30, 60 and 120 minute time points in the teasaponin treatment group compared to the HFD group (Figure 1F). However, blood glucose at 60 and 120 minutes in the teasaponin group was still higher than the LC group. In addition, we observed similar effects in the prevention of excessive weight gain, food intake and fat deposition, enlarged liver weight, and glucose intolerance (Fig. S1A – F), as well as improved recognition memory in the obese mice orally treated with teasaponin (Fig S1G).

3.2 Teasaponin restored leptin-pJAK2-pSTAT3 signaling in the prefrontal cortex of HFD fed mice. Several molecules, including JAK2-STAT3 and Akt-FOXO1/GSK, have been established as downstream mediators of leptin signaling in the hypothalamus [40-42]. Here we examined the leptin signaling pathways JAK2-STAT3 and Akt-FOXO1/GSK in the prefrontal cortex. An icv injection of leptin significantly increased pJAK2 in the prefrontal cortex of LC mice ($p<0.001$), but not in HFD fed mice ($p>0.05$, Fig 2A). However, with teasaponin treatment, the leptin injection significantly increased pJAK2 in HFD fed mice compared to saline injection ($p<0.05$). There was a similar response in the pSTAT3, downstream step of leptin/pJAK2 signaling in the LC, HFD and teasaponin-treated HFD mice (Fig 2B). Leptin significantly increased pSTAT3 by 42% in the prefrontal cortex of LC mice, while in HFD fed mice pSTAT3 only increased by 16% after the leptin injection. Following chronic teasaponin treatment, pSTAT3 increased by 46% in response to leptin compared with the saline injection, suggesting that teasaponin improved leptin-pJAK2-pSTAT3 signaling in HFD fed mice. We also found that leptin significantly increased pAkt, pGSK3β and pFOXO1 in the prefrontal cortex of LC but not in HFD fed mice ($p<0.05$, Fig 2C-E). However, teasaponin treatment did not reverse impaired leptin-pAkt-pGSK3β/pFOXO1 signaling in HFD fed mice. After icv leptin injection, the BDNF level in the prefrontal cortex was significantly increased in LC mice ($p<0.05$), but not in HFD fed mice (Fig 2F). Importantly, with chronic teasaponin treatment, leptin increased BDNF levels compared to saline in the prefrontal cortex of HFD fed mice, suggesting that teasaponin treatment improved the leptin-induced increase in BDNF in the prefrontal cortex. However, teasaponin treatment did not significantly alter basal BDNF protein levels in HFD mice ($p>0.05$).

3.3 Teasaponin restored central leptin sensitivity, and improved hyperinsulinemia and hyperleptinemia in HFD fed mice. In the LC group, there was a significant decrease in energy intake and body weight gain 24 hours after icv leptin ($p<0.05$, Table 1). However, in the HFD group leptin did not significantly decrease 24 hour energy intake and body weight gain, indicating decreased central leptin sensitivity. Teasaponin treatment restored central leptin sensitivity in the HFD group, as leptin injections decreased energy intake and body weight gain compared with saline in teasaponin treated HFD fed mice ($p<0.05$). A significant reduction in plasma insulin levels (-38.9%, $p<0.05$) was also observed in response to the icv leptin in LC mice, but not in HFD fed mice (-28.2%, $p>0.05$). Teasaponin treatment significantly decreased hyperinsulinemia in HFD fed mice by decreasing baseline circulating insulin by 56.2% ($p<0.05$), while leptin injection did not further decrease plasma insulin in the teasaponin treatment group. teasaponin treatment significantly attenuated hyperleptinemia in HFD fed mice ($p<0.05$).

3.4 Palmitic acid decreased leptin-induced BDNF production which can be corrected by teasaponin in the cortical neurons. In cultured prefrontal cortical neurons, immunostaining with anti-BDNF antibody showed a granular distribution of BDNF in MAP2-positive neurons (Fig 3B). Leptin treatment significantly increased BNDF immunoreactivity by 64% ($p<0.001$, Fig 3A) and mRNA expression 2.5 fold ($p<0.05$, Fig 3D). However, following palmitic acid pre-treatment, leptin did not increase BDNF immunoreactivity or mRNA expression (Figs 3A and 3D). This suggests that palmitic acid inhibits leptin stimulation of BDNF in prefrontal cortex neurons. Importantly, for both the low and high doses of teasaponin treatment, leptin significantly increased BDNF immunoreactivity in cortical neurons (both $p<0.001$), even when neurons were exposed to palmitic acid (Fig 3A). Moreover, high (40 μM, $p<0.001$) but not low

(20 μM) dose teasaponin, increased basal BDNF immunoreactivity (Fig 3A and 3C). Teasaponin (40 μM) also induced a significant increase in BDNF mRNA ($p<0.05$), suggesting that the teasaponin-induced increase in BDNF is due to an increase in BDNF translation.

3.5 Teasaponin promoted neurite outgrowth in response to leptin in cultured prefrontal cortical neurons. We examined the effect of teasaponin on neurite outgrowth in cells treated with leptin and palmitic acid (Figs 4A and 4B). Morphological analyses showed that leptin significantly increased the average neurite length (Fig. 4D) and total neurite length per cell (Fig. 4E) in control cortical neurons, but not in palmitic acid treated neurons. Whereas with teasaponin pre-treatment, leptin increased average neurite length (45%, $p<0.05$, Fig. 4D) and total neurite length per cell (45%, $p<0.05$, Fig. 4E) compare to saline, suggesting teasaponin attenuated the effect of palmitic acid on leptin-induced neurite length. However, the neurite number per cell in cortical neurons was not affected by leptin, PA or teasaponin (Fig. 4F). Furthermore, leptin treatment significantly increased neurite branching (branches per neurite: +59%, Fig. 4G, branches per cell: +59%, Fig. 4H) compared with saline treatment in control neurons, but not in palmitic acid treated neurons. With teasaponin pretreatment, leptin significantly increased branches per neurite, but not branches per cell. The mRNA expression of MAP2, a neurite marker, was also examined by RT-PCR. Teasaponin prevented palmitic acid from inhibiting the leptin-induced stimulation of MAP2 mRNA (Fig 4I).

3.6 Teasaponin promoted synaptogenesis in response to leptin in cultured prefrontal cortical neurons. Immunofluorescence microscopy showed that both SYN (pre-synaptic marker) and PSD95 (post-synaptic marker) had a strongly punctuated expression around the

spine of neurites (Figs 5A and 5C). The RT-PCR results also show that leptin significantly increased SYN and PSD95 mRNA expression in cultured cortical neurons ($p<0.05$, Figs 5B and 5D). Palmitic acid had no effect on basal SYN and PSD95 mRNA expression. However, it significantly inhibited SYN and PSD95 mRNA expression in response to leptin. Teasaponin prevented the inhibitory effect of palmitic acid on the leptin-induced stimulation of PSD95 mRNA expression ($p<0.05$), but not SYN mRNA expression.

4 Discussion

Our results show that chronic teasaponin treatment enhanced object recognition memory, and improved leptin signaling and leptin-induced BDNF expression in the prefrontal cortex of HFD fed mice. Moreover, in cultured primary prefrontal cortical neurons, palmitic acid impaired the effect of leptin on BDNF, neurite growth and synaptogenesis markers, and teasaponin ameliorated these palmitic acid induced effects. Previously, both clinical and animal studies have shown that tea improves cognitive function [26, 27] and prevents impairments of learning and memory [43]. Furthermore, tea increases brain activity in the prefrontal cortex of healthy volunteers as assessed by functional MRI methods [44]. In the present study, treatment with teasaponin, an important active ingredient of tea, enhanced memory for object recognition in HFD fed mice. Therefore, the effect of teasaponin on memory may contribute to the ability of tea to improve cognitive function and memory, and increase brain activation in the prefrontal cortex, as described previously [26, 27, 44].

In the present study, central acute administration of leptin significantly increased its downstream signaling molecule, pJAK2 and pSTAT3 in the prefrontal cortex of control mice. In accordance

with our results, another study demonstrated that systemic administration of leptin activated STAT3 phosphorylation in cortical neurons, in a rat model of cerebral ischemia [45]. We also have demonstrated that leptin activates pAkt, pFOXO1 and pGSK3β, another leptin signaling pathway, in the prefrontal cortex of LC mice. In obesity plasma leptin is increased, which theoretically will prevent overeating and obesity. However, this is not the case as leptin resistance is a pathology of obesity [46]. Furthermore, obese subjects with hyperleptinemia have an increased prevalence of dementia and other neurodegenerative disease [47-49]. Therefore, it is unlikely that high plasma leptin levels will appropriately activate signaling pathways in the brain of obese individuals with leptin resistance. Indeed, our results showed that, in HFD fed obese mice, both the leptin-pJAK2-pSTAT3 and pAkt-pGSK3β/pFOXO1 signaling pathways were impaired in the prefrontal cortex. Importantly, teasaponin treatment corrected alteration of the leptin-pJAK2-pSTAT3 signaling pathway in the prefrontal cortex of HFD fed mice. It has been reported that leptin replacement improves cognition in leptin-deficient patients with a delay or decline of cognitive function [19]. The prefrontal cortex is important for cognitive control [6], and HFD reduces synaptic plasticity in this region [11] leading to learning and memory impairments [12]. Therefore, teasaponin may attenuate HFD induced impairments of leptin signaling in the prefrontal cortex, leading to improved recognition memory.

In the prefrontal cortex, BDNF promotes neuronal plasticity and neurogenesis, which are important for learning and memory [7, 50]. HFD diet reduced BDNF in the prefrontal cortex and impaired discrimination reversal learning in rats [7]. A continuous icv infusion of antisense BDNF oligonucleotide resulted in an impairment of spatial learning in rats, with a significant reduction of BDNF mRNA and protein levels in the brain [51]. In the clinic, it is reported that

BDNF levels in plasma are positively correlated to memory performance in patients with depression [22]. Leptin administration has been shown to increase BDNF expression in the hippocampus of mice, which can be blocked by pre-treatment with K252a, a blocker of the BDNF receptor [15]. In this study, we observed that leptin stimulated BDNF expression in the prefrontal cortex of control mice, but not in HFD fed obese mice. In cultured primary prefrontal cortical neurons, leptin increased BDNF mRNA expression and immunoreactivity, however, this effect was impaired by palmitic acid. Along with improving the leptin-pJAK2-pSTAT3 signaling pathway, our results demonstrated a beneficial effect of teasaponin on the upregulation of BDNF by leptin. These results suggest that teasaponin-induced activation of leptin signaling may have modulated BDNF expression, enhancing neuronal plasticity in the prefrontal cortex and contributing to an improvement in recognition memory.

It is known that BDNF-mediated synaptic plasticity is usually coordinated by the synaptogenic proteins, SYN and PSD-95 [52-55]. SYN, a presynaptic vesicle phosphoprotein, is a marker of presynaptic nerve terminal density which regulates synaptic function, vesicle fusion, and neurotransmitter release [56]. PSD-95 is an important regulator of synaptic strength and plasticity [57, 58]. It has been reported that the expression of synaptogenesis markers, SYN and PSD95 depends on BDNF processing [59, 60]. Consistent with this, along with increased BDNF levels, our findings demonstrated that leptin stimulated SYN and PSD95 mRNA expression *in vitro*. Furthermore, mRNA expression of the neurite outgrowth marker MAP2 increased in response to leptin in cultured prefrontal cortical neurons. It is well established that leptin has important effects on neurogenesis, synaptogenesis and dendritic morphology in the brain [15, 61]. Overall, these results suggest that leptin may promote neurogenesis and synaptic plasticity

via increasing BDNF, SYN and PSD95 in cortical neurons. It has been reported that a diet high in saturated fatty acids induces neuronal degeneration and inactivates the leptin signaling molecule STAT3 in the hypothalamus of mice [62]. We observed that the neurite outgrowth and synaptogenesis responses to leptin were impaired in the cultured primary prefrontal cortical neurons pre-treated with palmitic acid. This suggests that saturated fatty acids also induce a leptin insensitivity effect on neurite outgrowth and synaptogenesis in the prefrontal cortical neurons. We also found that teasaponin reversed this adverse effect of palmitic acid on leptin-induced BDNF, neurite outgrowth and post-synaptogenic protein PSD95. It is known that the leptin signaling molecules, JAK2 and STAT3, are distributed around the postsynaptic sites in the cerebral cortex [63]. This suggests that teasaponin may modulate leptin signaling, BDNF expression and post-synaptic function in the prefrontal cortex, leading to improved recognition memory.

Teasaponin belongs to a family of molecules named steroidal saponins or triterpene saponins [64]. Steroidal saponins have an amphiphilic nature and are able to intercalate into the plasma membrane, replacing membrane cholesterol and increasing membrane fluidity. Thus, this molecule could change the immediate environment of cell membrane proteins, such as the GABA receptor [28, 29]. However, it remains to be determined if teasaponin directly interacts with the leptin receptor. This study demonstrated that teasaponin increased leptin sensitivity in the prefrontal cortex of obese mice, and in prefrontal neurons treated with PA, as well as reversed the alterations of leptin downstream signaling molecules (pJAK2, STAT3, BDNF and PSD95) (Fig S2). Furthermore, it is reported that hyperleptinemia is required for the development of leptin resistance in diet-induced obese mice [65]. In our study, teasaponin

significantly attenuates hyperleptinemia in HFD fed mice, which may reduce the overstimulation of the leptin receptor, thereby improving downstream signaling.

In summary, the findings of our study demonstrate that teasaponin improves recognition memory in the HFD fed mice. Through *in vivo* and *in vitro* studies we revealed that leptin activated leptin-pJAK2-pSTAT3 and pAkt-pFOXO1/pGSK3β signaling and stimulated BDNF expression and in the prefrontal cortex. However, these prefrontal cortex responses are impaired when mice are fed a high-saturated fat diet, or neurons exposed directly to saturated fatty acid, i.e. palmitic acid in vitro. Teasaponin treatment ameliorates alteration of leptin-pJAK2-pSTAT3 and leptin-BDNF in the prefrontal cortex of HFD fed mice and improves hyperleptinemia. Treatment with teasaponin also promoted leptin's effect on neurite outgrowth and synaptogenesis in prefrontal cortical neurons. Since high fat diet-induced obesity has been implicated in the progression of neurodegenerative diseases, such as vascular dementia, teasaponin supplementation may have beneficial effects in attenuating the progression of cognitive decline in obese patients and reducing the risk of neurodegenerative diseases. The dose of teasaponin obtained by frequently drinking tea is significantly lower than the doses used in this rodent study. In order to reach the effect observed in this study, a supplemental delivery of teasaponin (oral or injection) would be needed. Clinical trials would also be required to determine the optimum dose in humans. Furthermore, the potential toxicity of long-term teasaponin exposure should be investigated before teasaponin supplementation can be recommended.

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Table 1. Central leptin sensitivity and plasma levels of leptin and insulin in experiment groups in response to icv leptin injection

	LC group		HFD group		HFD+TS group	
	saline	**leptin**	**saline**	**leptin**	**saline**	**leptin**
Central leptin sensitivity						
EI (kcal/24 h)	15.65±1.12	10.01±1.06[a]	18.99±1.10	14.55±1.41	15.48±1.01[b]	10.35±1.22[bc]
BWC (g/24 h)	-0.19±0.084	-1.05±0.13[a]	-0.13±0.12	-0.65±0.25	-0.19±0.09	-1.90±0.37 [bcd]
Insulin (ng/ml)	1.31±0.11	0.80±0.10[a]	3.61±0.59[a]	2.59±0.37	1.58±0.39[b]	1.29±0.12[b]
Leptin (ng/ml)	0.65±0.08	0.79±0.09	16.78±2.02[a]	14.14±2.41	2.52±0.33[bc]	4.55±0.59[bc]

Central leptin sensitivity: Energy intake (EI) and body weight change (BWC) for 24 hours after icv leptin injection. LC: lab chow diet fed mice, HFD: high-fat diet fed mice; HFD+TS: HFD fed mice with teasaponin treatment (TS). [a]$p<0.05$ vs LC/saline; [b]$p<0.05$ vs HFD/saline; [c]$p<0.05$ vs HFD/leptin; [d]$p<0.05$ vs. HFD/TS/saline. Data are presented as mean±SEM, n=7–8.

Figure legends:

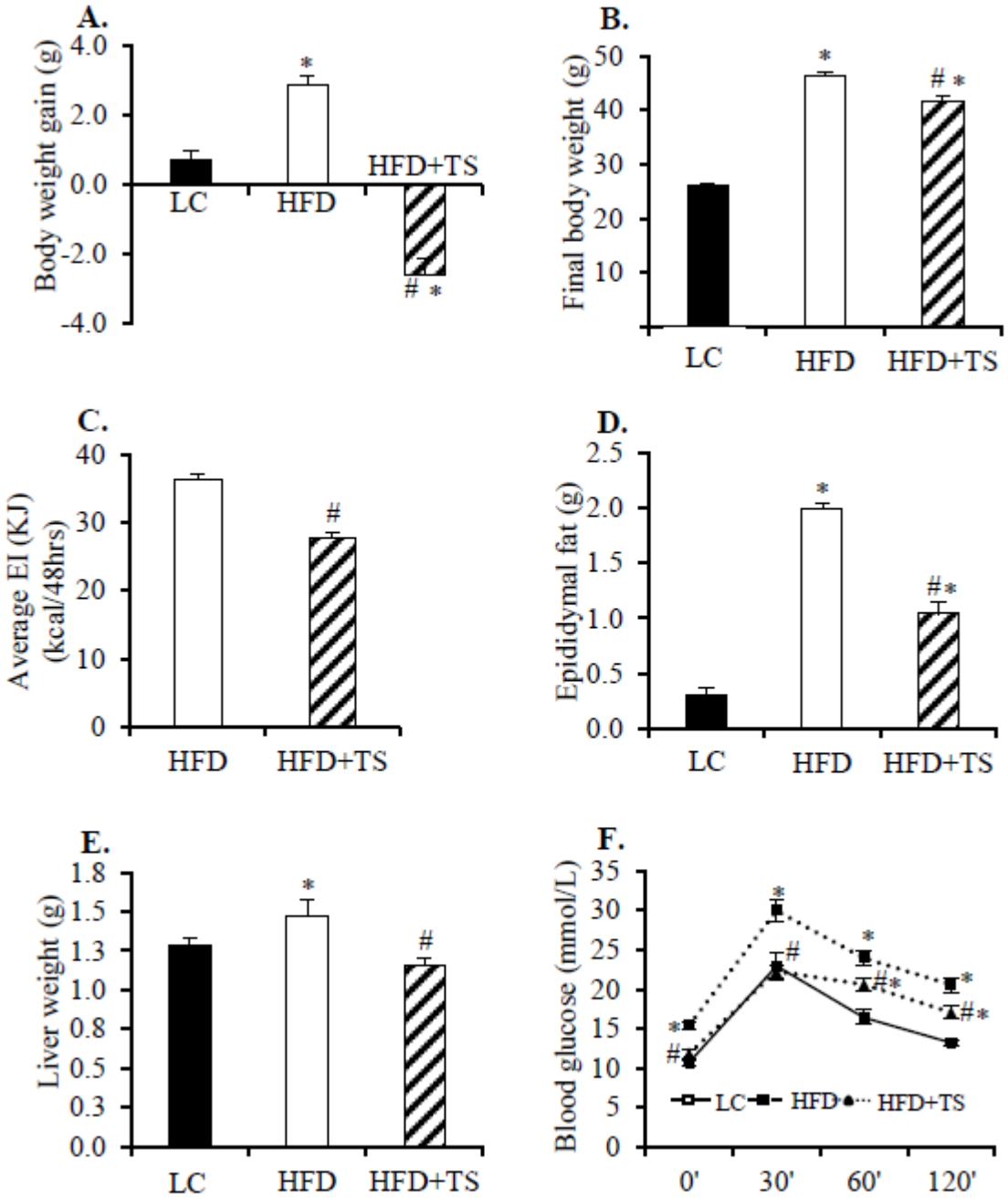
A.
Body weight gain (g)
4.0
2.0
0.0
-2.0
-4.0
LC
HFD
HFD+TS
B.
Final body weight (g)
50
40
30
20
10
LC
HFD
HFD+TS
C.
Average EI (KJ)
(kcal/48hrs)
40
30
20
10
0
HFD
HFD+TS
D.
Epididymal fat (g)
2.5
2.0
1.5
1.0
0.5
0.0
LC
HFD
HFD+TS
E.
Liver weight (g)
1.8
1.5
1.3
1.0
0.8
0.5
0.3
0.0
LC
HFD
HFD+TS
F.
Blood glucose (mmol/L)
35
30
25
20
15
10
5
0
LC
HFD
HFD+TS
0'
30'
60'
120'

Fig 1. Teasaponin treatment (TS, 10mg/kg, ip for 21 days) significantly improved metabolic parameters in mice fed high-fat diet (HFD) for 16 wks. Teasaponin reduced body weight gain (A), final body weight (B) average energy intake (C), epididymal fat (D) and liver weight (E) and glucose intolerance (F) in mice fed HFD for 16 wks. * $p<0.05$ vs. LC fed mice. $^{\#}$ $p<0.05$ vs. HFD fed mice. Data are presented as mean±SEM, n=10. LC: lab chow diet fed mice.

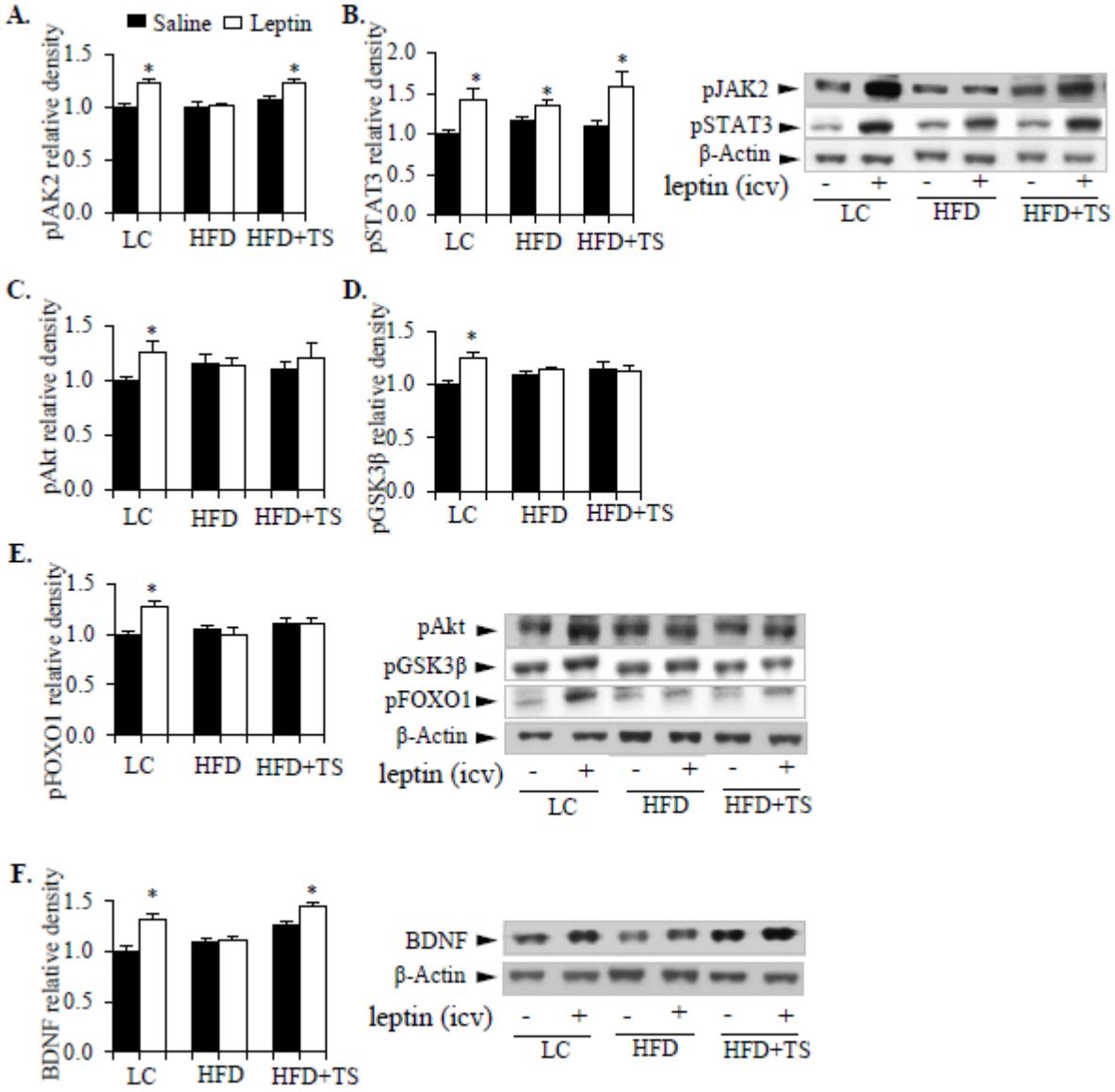
A.
Saline
Leptin
pJAK2 relative density
1.5
1.0
0.5
0.0
LC
HFD
HFD+TS
B.
pSTAT3 relative density
2.0
1.5
1.0
0.5
0.0
LC
HFD
HFD+TS
pJAK2
pSTAT3
β-Actin
leptin (icv)
- + - + - +
LC
HFD
HFD+TS
C.
pAkt relative density
1.5
1.0
0.5
0.0
LC
HFD
HFD+TS
D.
pGSK3β relative density
1.5
1.0
0.5
0.0
LC
HFD
HFD+TS
E.
pFOXO1 relative density
1.5
1.0
0.5
0.0
LC
HFD
HFD+TS
pAkt
pGSK3β
pFOXO1
β-Actin
leptin (icv)
- + - + - +
LC
HFD
HFD+TS
F.
BDNF relative density
1.5
1.0
0.5
0.0
LC
HFD
HFD+TS
BDNF
β-Actin
leptin (icv)
- + - + - +
LC
HFD
HFD+TS

Fig 2. Effect of teasaponin treatment (TS, 10mg/kg, ip for 21 days) on leptin-pJAK2-pSTAT3 signaling (A, B), pAkt-pGSK3β/pFOXO1 (C-E) and leptin-BDNF in the prefrontal cortex of high-fat diet (HFD) fed mice. *p<0.05 vs. saline in individual group. Data are presented as mean±SEM, n=8–9. LC: lab chow diet fed mice.

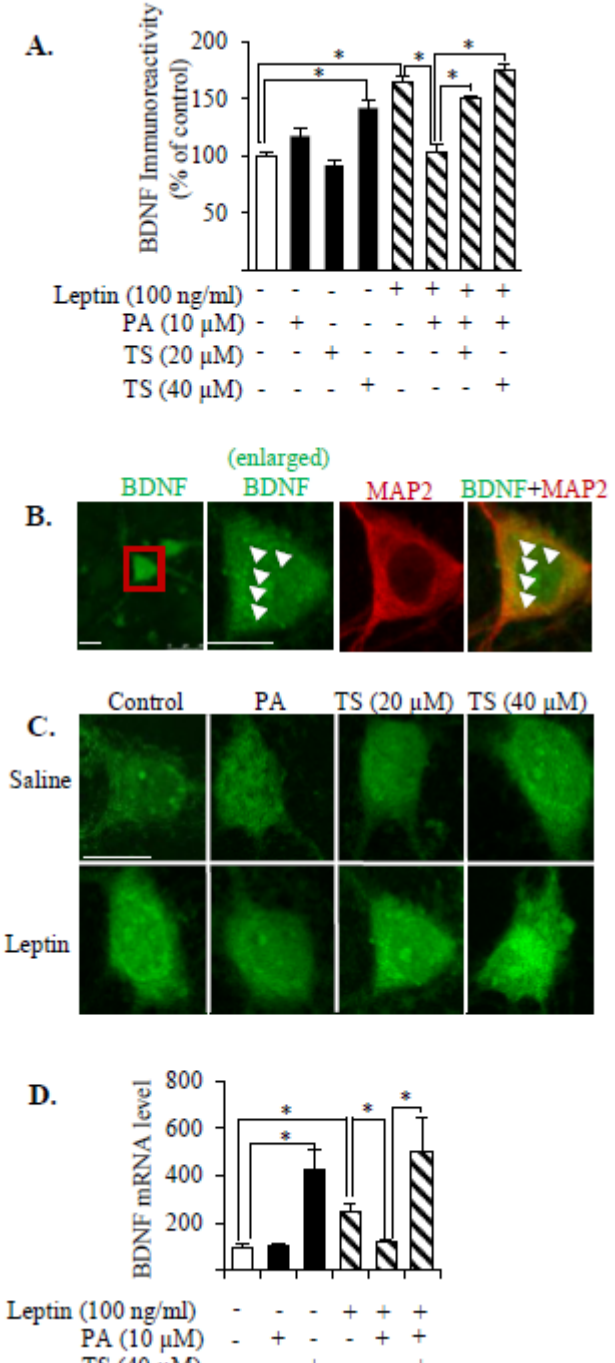

Fig 3. Palmitic acid (PA) decreased leptin-induced BDNF immunoreactivity (A, C) and mRNA expression (D) in cultured prefrontal cortical neurons, which can be corrected by teasaponin (TS). A and C: High dose of TS and leptin significantly increased BDNF immunoreactivity in cortical neurons. Pre-treatment with PA significantly inhibited leptin-induced BDNF. Both low and high dose of TS prevented PA from inhibiting the leptin-induced stimulation of BDNF. C: RT-PCR analysis of BDNF mRNA expression. B: Immunocytochemistry images showing the typical BDNF-containing vesicles (indicated by white arrowheads), taken by Confocal Microscope (Nikon).

For quantification of BDNF immunoreactivity, the mean intensity of BDNF fluorescence in 2–3 areas of the cell body per cell (approximately 100 μm^2) was measured using the software Image J 1.46r (http://rsbweb.nih.gov/ij/download.html).

Fifteen randomly selected neurons were analysed at ×100 magnification. Data expressed as mean±SEM (n=6, obtained from 6 independent culture wells). *p<0.05. Leptin (100 ng/ml) was applied to the neuron cultures 4 hours after 44 hour exposure to TS (20 or 40μM) or palmitic acid (10 μM). Scale bar =50 μm.

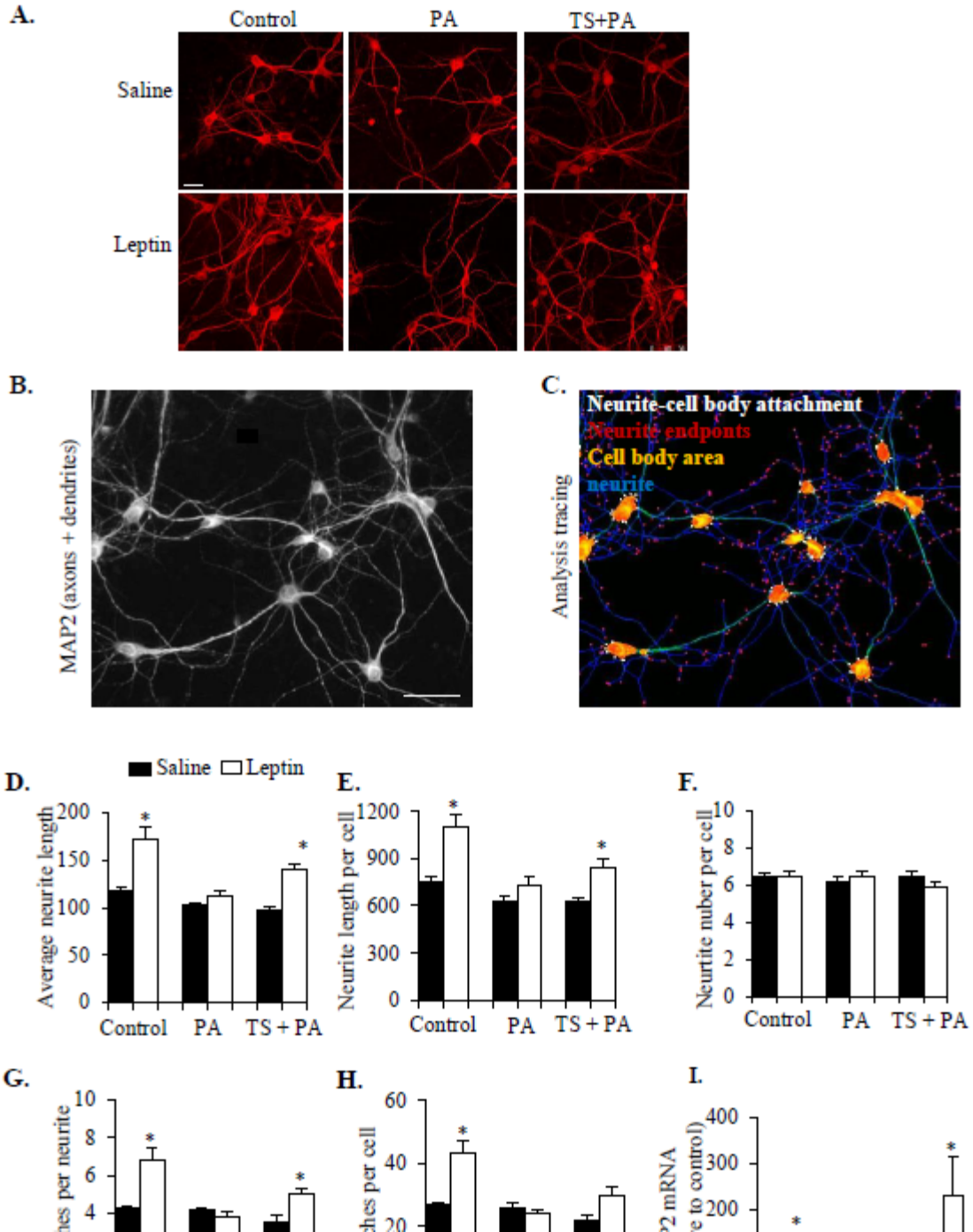
A.
Control
PA
TS+PA
Saline
Leptin
B.
MAP2 (axons + dendrites)
C.
Analysis tracing
Neurite-cell body attachment
Neurite endponts
Cell body area
neurite
D.
Saline
Leptin
Average neurite length
200
150
100
50
0
Control
PA
TS + PA
E.
Neurite length per cell
1200
900
600
300
0
Control
PA
TS + PA
F.
Neurite nuber per cell
10
8
6
4
2
0
Control
PA
TS + PA
G.
10
8
6
4
H.
60
40
20
I.
400
300
200

Fig 4. Effect of teasaponin (TS) and palmitic acid (PA) on the leptin-induced neurite outgrowth. A: represents fluorescence images obtained under Confocal Microscope (Nikon), scale bar = 50 μm; B: an example of MAP2 immunofluorescence staining image (8 bit) used for neuron morphology analysis, scale bar = 100 μm; C: an example of analysis tracing of neuron morphology quantification by using NeuriteQuant toolkit. All parameters (D-H) used were automatically analysed and calculated by the algorithm of NeuriteQuant in pixels, n=9; D: Average neurite length; E: Neurite length per cell; F: Neurite number per cell; G: Branches per neurite; H: Branches per cell; I: MAP2 mRNA expression measured by qRT-PCR (n=5-7). *p<0.05 vs. saline in individual group. Data are presented as mean ± SEM. Leptin (100 ng/ml) was applied to the neuron cultures 4 hours before 44 hour exposure to TS (20 or 40μM) or palmitic acid (10 μM).

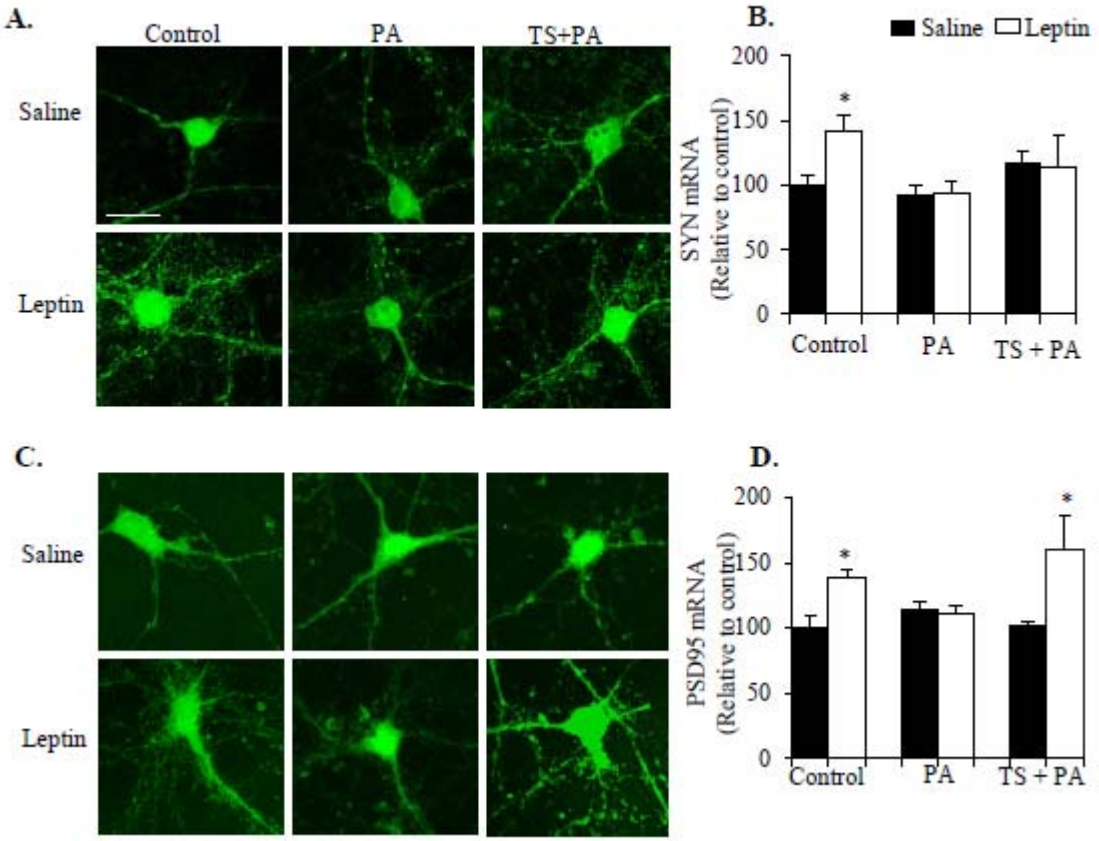

Fig 5. Effect of teasaponin (TS) and palmitic acid (PA) on the leptin-induced synaptogenesis markers, SYN (A and B) and PSD95 (C and D), in cultured primary prefrontal cortical neurons. A and C represent fluorescence images obtained under Confocal Microscope (Nikon). B and D represent RT-PCR analysis of SYN and PSD95 mRNA expression. *p<0.05 vs. saline in individual group. Data are presented as mean±SEM, n=5–6. Cultured primary prefrontal cortical neurons were pre-treated with teasaponin (40 μM) for 5 hours and PA (10 μM) for 4 hours and then treated with leptin (100 ng/ml) for 44 hours. Scale bar=50 μm.

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Conflict of interest Statement: The authors declare no conflict of interest.

SUPPLEMENTARY DATA

Table S1. The antibodies used in this study

Peptide/protein target	Name of Antibody	Manufacturer, catalog #	Species raised in monoclonal or polyclonal	Dilution used
BDNF	BDNF (H-117)	Santa Cruz Biotechnology, sc-20981	Rabbit, Polyclonal	1:400
TrkB 145 and 95	TrkB (H-181)	Santa Cruz Biotechnology, sc-8316	Rabbit, Polyclonal	1:500
pTrkB 95	Anti-phospho-Trk B (pTyr705)	Sigma-Aldrich, SAB4503786	Rabbit, Polyclonal	1:500
p-JAK2	p-JAK2 (Tyr 1007/Tyr 1008)	Santa Cruz Biotechnology, sc-21870	Goat, Polyclonal	1:500
p-STAT3	Phospho-Stat3 (Tyr705) (D3A7) XP® Rabbit mAb	Cell Signaling Technology, #9145	Rabbit, Monoclonal	1:2000
pAkt	p-Akt1/2/3 (Ser 473)-R	Santa Cruz Biotechnology, sc-7985-R	Rabbit, Polyclonal	1:500
pGSK3β	Phospho-GSK-3β (Ser9)(5B3) Rabbit mAb	Cell Signaling Technology, #9323	Rabbit, Monoclonal	1:1000
BDNF	BDNF Antibody (H-117)	Santa Cruz Biotechnology, sc-20981	Rabbit, Polyclonal	1:100
MAP2	Monoclonal Anti-MAP2 antibody	Sigma-Aldrich, #M4403	Mouse, Monoclonal	1:500
SYN	Anti-Synaptophysin antibody	Sigma-Aldrich, SAB4502906	Rabbit, Polyclonal	1:100
PSD95	Rabbit anti-PSD-95	Life Technologies, #51-6900	Rabbit, Polyclonal	1:500

Table S2. The primers used in qPCR for mRNA measurement of neurogenesis markers

GENE	Forward primer	Reverse primer	NCBI reference
BDNF	GGGTCACAGCGGCAGATAAA	GCCTTTGGATACCGGGACTT	NM_001285416.1
MAP2	ATGAAGGAAAGGCACCACAC	AATAGGTGCCCTGTGACCTG	NM_001039934.1
SYN	GAACAAGTACCGAGAGAACAACAA	GGTCAGTGGCCATCTTCACA	NM_009305.2
PSD95	GGTGACGACCCATCCATCTTTATC	CGGACATCCACTTCATTGACAAAC	NM_007864.3
γ-actin	GCTAACAGAGAGAAGATGACG	CAGATGCATACAAGGACAGC	NM_009609.2

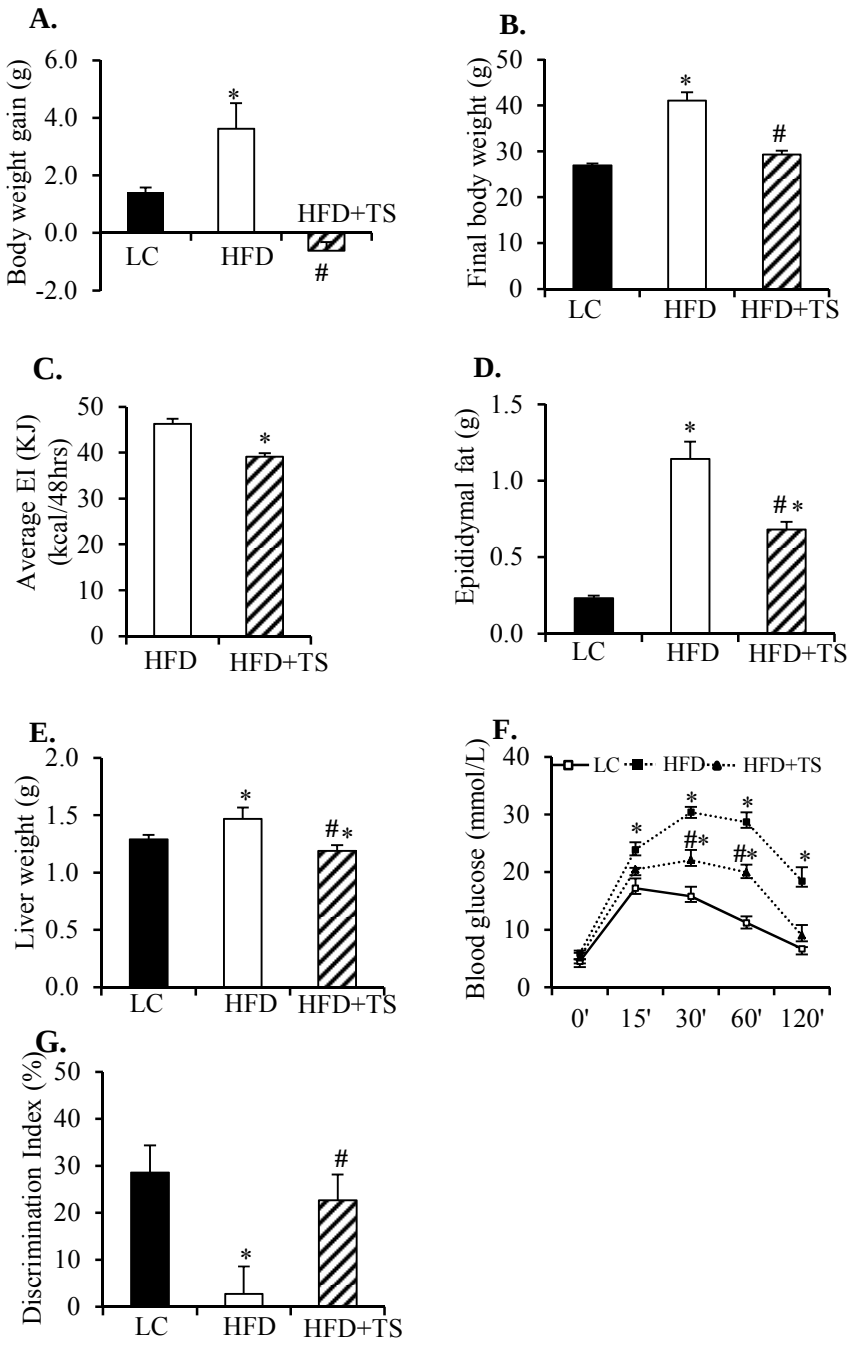

Fig S1. Teasaponin treatment (TS, 0.5% in high-fat diet for 6 weeks) significantly improved metabolic parameters and recognition memory deficits in mice fed high-fat diet (HFD) for 8 wks. Teasaponin prevented body weight gain (A), final body weight (B), average energy intake (C), epididymal fat (D) and liver weight (E) and glucose intolerance (F) in mice fed HFD for 8 wks. (G) Teasaponin attenuated HFD-induced decline in discrimination index reflecting recognition memory in the novel object recognition test in mice. Discrimination Index = (Novel Object Exploration Time / Total Exploration Time) – (Familiar Object Exploration Time / Total Exploration Time) × 100. * p<0.05 vs. LC fed mice. $^{\#}$ p<0.05 vs. HFD fed mice. Data are presented as mean±SEM, n=10. LC: lab chow diet fed mice.

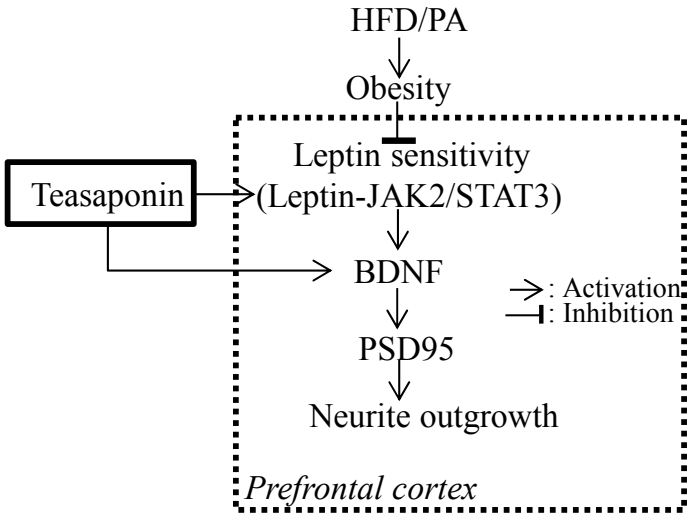

Fig S2. Teasaponin improves leptin sensitivity through actions on leptin-JAK2/STAT3, BDNF and PSD95 molecules in the prefrontal cortex. HFD: high-fat diet; PA: palmitic acid.